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71 Applicant: WARNER-LAMBERT COMPANY
201 Tabor Road
Morris Plains New Jersey 07950(US)

72 Inventor: Hokanson, Gerard Clifford
2408 Antietam
Ann Arbor Michigan 48105(US)

72 Inventor: Schaumburg, John Phillip
1205 Westmoorland
Ypsilanti Michigan 48197(US)

72 Inventor: French, James Clark
3150 Rumsey
Ann Arbor Michigan 48105(US)

72 Inventor: Tunac, Josefino Ballesteros
5284 Collington Drive
Troy Michigan 48098(US)

74 Representative: Jones, Michael Raymond et al,
HASELTINE LAKE & CO. Hazlitt House 28 Southampton
Buildings Chancery Lane
London WC2A 1AT(GB)

54 An antibiotic compound and its production.

57 A purified isolate of an actinomycete identified as ATCC 39366 is capable of producing the antimicrobial compound CL-1957B which also exhibits antitumor properties.

The antimicrobial compound CL-1957B is produced by cultivating isolate ATCC 39366 under aerobic conditions in a culture medium containing assimilable sources of carbon and nitrogen until a substantial quantity of the CL-1957B compound is produced, and subsequently isolating the compound.

The antibiotic compound CL-1957B, its pharmaceutically acceptable salts, and pharmaceutical compositions comprising this substance together with a pharmaceutically acceptable carrier is also disclosed, as are methods of treating microbial infections and tumors in mammals, employing these pharmaceutical compositions.

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AN ANTIBIOTIC COMPOUND AND ITS PRODUCTION

The present invention relates to an antibiotic compound demonstrating antitumor activity, designated CL-1957B and its pharmaceutically acceptable salts,
5 to a process for the production of said compound, and to a purified isolate of an actinomycete capable of producing this compound.

More particularly, the process of producing the CL-1957B antibiotic compound relates to an aerobic
10 fermentation process using a purified isolate of an actinomycete, identified as isolate ATCC 39366.

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In accordance with one aspect of the present invention, there is provided a purified isolate of an actinomycete having the identifying characteristics of ATCC 39366 which is capable of producing the
5 antibiotic compound CL-1957B.

In another aspect of the invention, there is provided a process for producing CL-1957B by cultivating the isolate of actinomycete identified as ATCC 39366 under aerobic conditions in a medium
10 containing assimilable sources of carbon and nitrogen until a substantial quantity of CL-1957B is produced, and subsequently isolating the compound.

In accordance with another aspect of the invention, there are provided the antibiotic compound
15 CL-1957B and its pharmaceutically acceptable salts, which compounds exhibit both antimicrobial and antitumor properties.

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In another aspect of the present invention, there are provided pharmaceutical compositions comprising at least one CL-1957B compound, its pharmaceutically acceptable salts and, optionally, additional anti-
5 microbial and/or antitumor compounds together with a pharmaceutically acceptable carrier.

The compound of the present invention is useful in a method of treating microbial infections in a mammal which method comprises administering
10 an effective amount of the compound CL-1957 or a pharmaceutically acceptable salt thereof in combination with a pharmaceutically acceptable carrier.

The compound of the present invention is also useful in a method of treating tumours in mammals which method comprises administering
15 an effective amount of the compound CL-1957B or a pharmaceutically acceptable salt thereof in combination with a pharmaceutically acceptable
20 carrier.

FIGURES 1a, 1b, 1c, and 1d are the ultraviolet, infrared, 360 MHz proton magnetic resonance, and 90.5 MHz ^{13}C nuclear magnetic resonance spectra, respectively, of the compound
25 designated CL-1957B.

In accordance with the present invention, the
30 CL-1957B antibiotic compound is produced by cultivating a selected isolate of actinomycete, isolate ATCC 39366, under artificial conditions until a substantial quantity of CL-1957B is formed, and subsequently isolating the compound.

35 The strain of actinomycete suitable for the purpose of this invention was found in a soil sample collected in Pennsylvania, USA. This microorganism

was isolated from the soil sample using a suitable agar plating medium, one containing salts such as potassium phosphate, magnesium sulfate, and ferrous sulfate, and carbon sources such as glycerol and asparagine. The strain of microorganism was plated onto the agar medium and, once plated, was incubated at a favorable temperature, particularly 45°C, to allow for the development of the soil microorganisms.

The CL-1957B producing organism that was isolated from the soil sample by the agar plating technique is an unidentified isolate of actinomycete and has been deposited with the American Type Culture Collection, Rockville, Maryland 20852, where it is being maintained in their permanent culture collection as ATCC 39366. This organism, which produces CL-1957B, is also being maintained as a dormant culture in lyophile tubes, cryogenic vials, and in soil tubes in the Warner-Lambert/Parke-Davis Culture Collection, 2800 Plymouth Road, Ann Arbor, Michigan 48105, where it is designated as culture WP-2053.

The compound CL-1957B, which demonstrates both antimicrobial and antitumor properties, is produced by isolate ATCC 39366 during aerobic fermentation under controlled conditions. The fermentation medium consists of sources of carbon, nitrogen, minerals, and growth factors. Examples of carbon sources are glycerol and various simple sugars, such as glucose, mannose, fructose, xylose, ribose, or other carbohydrate-containing compounds such as dextrin, starch, cornmeal, and whey. The normal quantity of carbon source materials in the fermentation medium varies from about 0.1 to about 10 weight percent.

Nitrogen sources in the fermentation medium are organic, inorganic, or mixed organic-inorganic material. Examples of such materials are cottonseed

meal, soybean meal, corn germ flour, corn steep liquor, distillers dried solubles, peanut meal, peptonized milk, and various ammonium salts.

The addition of minerals and growth factors are also helpful in the production of the CL-1957B compound. Examples of fermentation medium mineral additives include potassium chloride, sodium chloride, ferrous sulfate, calcium carbonate, cobalt chloride, and zinc sulfate. Sources of growth factors include various yeast and milk products.

The preferred method for producing the CL-1957B compound is by submerged culture fermentation. According to this embodiment of the invention, the fermentation ingredients are prepared in solution or suspension and the mixture subsequently sterilized by autoclaving or steam heating. The pH of the aqueous medium is adjusted to preferably between about pH 4 and about pH 8 and the mixture cooled following sterilization to a temperature between about 16°C to about 45°C. The cooled, sterile fermentation medium is inoculated with the organism and thereafter fermentation is carried out with aeration and agitation.

In the submerged culture method, fermentation is carried out in shake-flasks or in stationary tank fermentors. In shake-flasks, aeration is achieved by agitation of the flasks to bring about mixing of the medium with air. In stationary tank fermentors, agitation is provided by impellers which may take the form of disc turbines, vaned discs, open turbine or marine propellers. Aeration is accomplished by injecting air or oxygen into the agitated mixture.

Adequate production of the CL-1957B compound is normally achieved under these conditions after a period of about two to ten days.

In an alternative embodiment, the CL-1957B compound may also be produced by solid state fermentation of the microorganism.

CHARACTERISTICS OF ATCC 39366

- 5 Culture characterization studies were carried out on the microorganism designated ATCC 39366 (CL 1957 producer) and the results were compared to the published data on Streptomyces sp 81-484 (kazugamycin producer) and Streptomyces ATS 1287
10 (leptomycin producer).

Cultural and morphological characteristics of ATCC 39366 are shown in Tables A, B and C.

TABLE A

- Carbohydrate Utilization Pattern of ATCC 39366
15 as Compared with Published Data for Streptomyces
ATS 1287 and Streptomyces 81-484

	Carbon Substrate	ATCC 39366 (CL 1957)	Streptomyces ATS 1287 (Leptomycin)	Streptomyces 81-484 (Kazugamycin)
20	1-arabinose	-	-	-
	d-fructose	+	+	-
	d-galactose	+	+	ND
	d-glucose	+	+	ND
25	glycerol	+	ND*	ND
	i-inositol	+	-	+
	inulin	-	ND	ND
	lactose	-	ND	ND
	maltose	-	ND	ND
30	d-mannitol	-	-	-
	d-mannpse	+	ND	ND
	raffinose	+	+	-
	rhamnose	+	+	+
	salicin	+	-	ND
35	sucrose	-	-	-
	d-xylose	+	-	-
	control	-	-	-

* ND = not done.

TABLE B

Physiological Characterization of ATCC 39366
as Compared to Published Data on Streptomyces
ATS 1287 and Streptomyces 81-484

Reaction	ATCC 39366 (CL 1957)	Streptomyces ATS 1287	Streptomyces 81-484
Melanine Pigment	None	None	None
Other Pigment	None	None	Positive
Nitrate Reduction	Positive	Negative	Negative
Gelatin Liquefaction	Positive	Negative	Negative
Milk Coagulation	Negative	Negative	Negative
Milk Preparation	Positive	Weak Preparation	Doubtful

ATCC differs from 81-484 in the utilization
of d-fructose, raffinose and d-xylose. It differs
in nitrate reduction-gelatin liquefaction.

ATCC 39366 belongs to the grey series and 81-484
belongs to the yellow series.

TABLE C

Cultural and Morphological Characteristics of
ATCC 39366 as Compared to Published Data on
Streptomyces ATS 1287 and Streptomyces 81-484

5	Categories	ATCC 39366 (CL 1957)	Streptomyces ATS 1287 (Leptomycin)	Streptomyces 81-484 (Kazugamycin)
	<u>Aerial</u>			
10	Yeast extract- Malt extract- ISP 2	slate (13 ih)	grey	ivory
	Otameal agar ISP 3	none	grey	pale yellow
15	Inorganic salts starch agar ISP 4	pewter (13 fe)	thin	ivory
	Glycerol asparagine agar ISP 5	near grey (7ml)	grey	brownish white
20	<u>grouping</u>	grey series	grey series	yellow series
	<u>Reverse</u>			
	ISP 2	mustard gold (2 pg)	olive buff	pastel yellow
25	ISP 3	olive (2 pl)	none	pale yellow
	ISP 4	colourless	none	ivory
	ISP 5	light wheat (2 ea)	olive buff	yellowish grey
	<u>Spores</u>			
30		spiral spore chains; >10 spores/chain; smooth spore surface; cylindrical spore shape.	spiral spore chains; >10 spores/chain; smooth spore surface; not described.	rectus spore chain; >6 spores/chain; smooth spore surface; cylindrical spore shape.
35	Cell Wall DAP Whole cell sugar	LL- arabinose	not reported not reported*	LL- not reported

* Classified as Streptomyces indicating no major sugars were found.

The following examples are provided to enable one skilled in the art to practice the present invention and are merely illustrative thereof. They are not to be viewed as limiting the scope of the invention as defined by the appended claims.

Fermentative Production of the CI-1957B Compound

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EXAMPLE 1

The culture of actinomycete (ATCC 39366) of the present invention, following its isolation from the agar plate, was transferred to an agar slant employing CIM 23 medium and incubated at 28°C for 7 to 14 days.

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TABLE I

Formulation of CIM 23 Medium

	Amidex corn starch	10 g
	N-Z amine, type A	2 g
	Beef Extract (Difco)	1 g
20	Yeast Extract (Difco)	1 g
	Cobalt chloride pentahydrate	20 mg
	Agar	20 g
	Distilled water	1000 ml

EXAMPLE 2

A portion of the microbial growth from the agar slant was used to inoculate an 18-mm x 150-mm test tube containing 5 ml of SD-05 seed medium. The inoculated seed was shaken at 24°C, 170 rpm, for three to four days.

TABLE II

Formulation of SD-05 Seed Medium

	Amberex 1003 (Amber Laboratories)	0.5 %
10	Glucose monohydrate (Cerelease)	0.1 %
	Dextrin-Amidex B 411 (Corn Products)	2.4 %
	N-Z Case (Humko Sheffield)	0.5 %
	Spray-dried meat solubles (Daylin Labs)	0.3 %
	Calcium carbonate	0.2 %

15

EXAMPLE 3

A 1-ml portion of the microbial growth of Example 2 was transferred to a 185-ml shake-tube containing 25 ml of SM-57 screening medium.

TABLE III

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Formulation of SM-57 Screening Medium

	Sucrose	1.5 %
	Lactose	1.0 %
	Peptonized milk	0.65%
	Fish meal	0.35%
25	Torula yeast	0.25%

The inoculated shake-tube was incubated at 24°C for four days with shaking (170 rpm gyratory shaking, 5 cm throw). Production of the CL-1957B compound was observed for the first time in this broth.

5 To confirm the fermentation activity of the microorganism, a second 50-ml batch of SM-57 screening medium, contained in a 300-ml baffled shake-flask, was inoculated with 2 ml of microbial seed from Example 2. This mixture was incubated at 24°C for four days with
10 shaking (170 rpm gyratory shaking, 5 cm throw). After four days, the fermentation beer was granular to mycelial in appearance, and the pH was in the range of pH 5.5-6.0.

The antitumor activity of this fermentation broth
15 was assayed at a dilution of 1:100 versus L1210 mouse leukemia cells grown in tissue culture. The assay technique is fully described in Cancer Chemotherapy Reports, Part 3, Vol. 3, No. 2 (1972), Deran, Greenberg, MacDonald, Schumacher, and Abbott. A broth
20 which gave L1210 leukemia cell growth rates of 0 to 35%, compared with the growth of these cells under control conditions, was considered active, 0%, most active. The observed activities of the fermentation broth of Example 3 are given in Table IV.

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TABLE IV

Antitumor Activity of Fermentation Broth from
Example 3 (As Measured Against L1210 Mouse
Leukemia Cells)

	Sample	% L1210 Cell Growth
30	Broth from shake-tube	11
	Broth from shake-flask	6

The crude fermentation beer from Example 3 was also tested for antimicrobial activity against several microorganisms employing the agar-disc method. The crude beer was found to be active against

- 5 Agrobacterium tumefaciens, Alcaligenes viscolactis,
Bacillus subtilis, Branhamella catarrhalis, Escherichia coli, Micrococcus luteus, and Micrococcus lysodeikticus.

EXAMPLE 4

- 10 Two 2-liter shake-flasks, each containing 300 ml of SM-57 screening medium, were inoculated with 12 ml of microbial seed. The flasks were incubated for four days at 24°C with shaking (170 rpm gyratory shaking, 5 cm throw).

- 15 The fermentation beer from the two flasks was pooled and tested for antitumor activity against both L1210 mouse leukemia cells grown in tissue culture and P388 murine lymphocytic leukemia in vivo. Both tests were conducted in accordance with the methods detailed
 20 in Cancer Chemotherapy Reports, Part 3, Vol. 3, No. 2 (1972) referenced above.

- The crude beer was observed to limit L1210 cell growth to 6% in vitro. The results of the P388 in vivo tests appear in Table V. The data are given
 25 there in terms of % T/C values where:

$$\% \text{ T/C} = \frac{\text{median survival time of treated mice}}{\text{median survival time of control mice}} \times 100$$

TABLE V

Antitumor Activity of Fermentation Broth from
Example 4 (As Measured Against P388 Murine
Lymphocytic Leukemia In Vivo)

5	Dilution of Fermentation		
	Beer	Test 1	Test 2
	Undiluted	Toxic	--
	1:2	66 (Toxic)	59 (Toxic)
	1:4	146	126
10	1:8	---	140
	1:16	---	117

EXAMPLE 5

Culture suspension (1 ml) from a cryogenic vial was thawed and aseptically transferred to a 2-liter baffled flask containing 600 ml of SD-05 seed medium. The inoculated flask contents were incubated for 72 hours at 24°C with shaking (130 rpm gyratory shaking, 5 cm throw).

After 72 hours, the contents of the seed flask were transferred aseptically to a 30-liter jar fermentor containing 16 liters of SD-05 seed medium. The inoculated jar contents were incubated for 24 hours at 24°C while being stirred at 300 rpm and sparged with air at a rate of 1 vol/vol/min.

EXAMPLE 6

Three 30-liter stirred-jars, each containing 16 liters of PM-10 production medium, were sterilized by autoclaving for 40 minutes at 121°C. The fermentors and contents were cooled and each was

inoculated with about 800 ml of the microbial growth from Example 5. The inoculated production jars were incubated for six days at 24°C while being stirred at 300 rpm and sparged with air at a rate of 1 vol/vol/5 min. Dow Corning "C" antifoam agent was used to control foaming.

TABLE VI

Formulation of PM-10 Production Medium

	Maltose	1.5 %
10	Glucose monohydrate	1.0 %
	Cotton seed meal (Pharmamedia)	0.75%
	Corn meal	0.4 %
	Torula yeast	0.25%
	Note: pH adjusted to 6.5 with NaOH	

15 The production of the CL-1957B compound was monitored throughout the fermentation cycle by assay against L1210 mouse leukemia in vitro, and by measuring antimicrobial activity against several microorganisms. In addition, such fermentation
20 parameters as pH and percent sedimentation were recorded throughout the fermentation cycle. The data are presented in Table VII.

TABLE VII

Fermentation Time pH (Hours)	% Sedimentation (Growth)	Observed Bioactivity								
		Inhibition of Growth of Microorganism Inhibition Zone Diameter (mm) (Using 12.7 mm discs)			Percent Growth of L1210 Mouse Leukemia Cells at Given Dilution					
		<u>E. coli</u>	<u>B. subtilis</u>	<u>M. luteus</u>	1:100	1:500	1:1000	1:2500	1:5000	
0	6.3	---	---	---	---	---	---	---	---	---
24	6.4	---	---	---	NA*	---	---	---	---	---
48	5.9	21	23	15	5.7	1.6	---	---	---	---
69	5.2	19	23	16	6.2	0.4	---	---	---	---
96	5.15	19	20	16	5.7	0	---	---	---	---
120	6.0	19	19	17	---	0	1.5	2.8	2.9	2.9
144	6.1	21	19	17	---	0	1.3	2.9	3.2	3.2

NA = Not active

*NA = Not active

Example 7

A 1-ml portion of a cryogenically preserved culture of isolate ATCC 39366 was used to inoculate 600 ml of SD-05 seed medium contained in a 2-liter baffled shake-flask. The inoculated shake-flask contents were incubated for 71 hours at 24°C with shaking (130 rpm gyratory shaking, 5 cm throw).

The microbial growth from the 2-liter flask was used to inoculate 16 liters of SD-05 seed medium contained in a 30-liter stirred jar fermentor. The inoculated fermentor contents were incubated at 24°C for 24 hours while being stirred at 300 rpm and sparged with air at a rate of 1 vol/vol/min.

A 200-gallon (757-liter) fermentor containing 160 gallons (606 liters) of PM-10 production medium was sterilized by heating with steam for 40 minutes at 121°C. The fermentor and its contents were cooled to 24°C and inoculated with about 15 liters of the microbial growth from the 30-liter stirred jar fermentor. The inoculated production medium was incubated at 24°C for five days with stirring at 155 rpm while being sparged with air at a rate of 0.75 vol/vol/min. Dow Corning "C" antifoam agent was added as needed to control foaming of the fermentation medium.

The production of the CL-1957B compound was monitored throughout the fermentation cycle using the L1210 mouse leukemia cell assay, by measuring the antimicrobial activity of the fermentation beer against Micrococcus luteus and Bacillus subtilis, and by such fermentation parameters as pH and percent sedimentation. The data appear in Table VIII.

TABLE VIII

Fermentation Time pH (hours)	Sedimentation (Growth)	Observed Bioactivity		Percent Growth of L1210 Mouse Leukemia Cells at Given Dilution		
		Inhibition of Growth of Microorganism Inhibition Zone Diameter (mm) (Using 12.7 mm discs)	Micrococcus luteus	Bacillus Subtilis	1:100	1:500
0	6.35	---	---	---	---	---
26	6.65	4.7	0	0	NA ^a	---
52	6.10	7.4	14.0	20.5	5.0	4.5
72	6.0	8.7	16.5	21.5	6.8	3.8
96	5.9	11.3	16.5	23.5	---	5.5
116	6.0	14.7	18.0	20.0	---	0
NA = Not active					0	3.8
						3.1

The crude beer was harvested, and the CL-1957B compound isolated as described below.

Chemical Isolation of the CL-1957B Compound

EXAMPLE 8

5 Fermentation beer as prepared above in
Example 7 was adjusted to pH 3.5 with sulfuric acid
and mixed for one hour with ethyl acetate (227
liters). Celite 545 (11.4 kg) was added and the mix-
10 ture filtered through a 46-cm plate-and-frame filter
press. The filtrate was allowed to stand to let the
lower aqueous phase separate from the ethyl acetate
extract. The filter cake was washed with ethyl
acetate (132 liters) and the wash, after dilution with
76 liters of fresh ethyl acetate, was used to extract
15 the separated aqueous layer from above. After
allowing the mixture to settle, the aqueous and
organic layers from the second extract were separated
and the aqueous layer extracted a third time with
fresh ethyl acetate (189 liters). The three organic
20 layers were combined and washed with deionized water
(95 liters). The mixture was allowed to settle and
the water wash separated. The upper ethyl acetate
layer (529 liters) was concentrated in vacuo to 31
liters and then concentrated further with the
25 displacement of ethyl acetate by methanol to yield 4.5
liters of a methanolic concentrate. This concentrate,
diluted with one-tenth volume of water, was extracted
two times with four liter portions of petroleum ether
(bp 30°-60°C) and then concentrated to approximately
30 500 ml. Continued concentration with displacement of
methanol by water yielded approximately 400 ml of an
aqueous suspension which was extracted three times
with 400 ml portions of ethyl acetate. The ethyl
acetate extracts were combined, dried over anhydrous

sodium sulfate, filtered, concentrated to a small volume, and then mixed with 250 grams of a mixture of silicic acid and Celite 545 (1:1). The resulting slurry was evaporated in vacuo to a dry solid which

5 was slurried with dichloromethane (300 ml), and added to the top of a column containing 4 kg of a mixture of silicic acid and Celite 545 (1:1) packed in dichloromethane. The column was washed with dichloromethane (16 liters) and then eluted with

10 dichloromethane-methanol (99:1, 14 liters), dichloromethane-methanol (98:2, 20 liters) and dichloromethane-methanol (96:4, 20.5 liters). Concentration of the dichloromethane-methanol (96:4) eluates afforded a viscous oil containing CL-1957B.

Purification of CL-1957B

EXAMPLE 9

The crude CL-1957B fraction from silicic acid-Celite chromatography was triturated two times with 500-ml portions of n-heptane. The heptane insoluble material (18.9 g) was chromatographed over 750 g of silica gel 60 (40-60 μ m particle size, E. Merck Reagents) deactivated with 1% water, contained in a 6 cm [i.d.] X 60 cm column. The column was eluted with dichloromethane-methanol (95:5), collected in nine 500-ml fractions. Fractions six and seven, which contained most of the CL-1957B (as determined by HPLC and TLC assay), were combined and concentrated to dryness to yield 4.6 g of partially purified residue. Further purification was effected by chromatography over 1.9 kg of C₁₈-silica gel (Sepralyte C-18, 40 μ m particle size, Analytichem International) contained in a stainless steel column (7cm [i.d.] X 85 cm). The column was eluted with methanol-water (7:3), collected in sixteen one-liter fractions. Fractions eleven through fifteen, which contained most of the CL-1957B (by HPLC assay), were combined and concentrated to yield 1.7 g of purified CL-1957B as a light tan solid foam.

The chemical and physical properties of CL-1957B appear in Table IX and the ultraviolet, infrared, 360 MHz proton magnetic resonance, and 90.5 MHz ¹³C nuclear magnetic resonance spectra of the compound appear as Figures 1a, 1b, 1c, and 1d, respectively.

TABLE IX

Chemical and Physical Properties of Cl-1957b

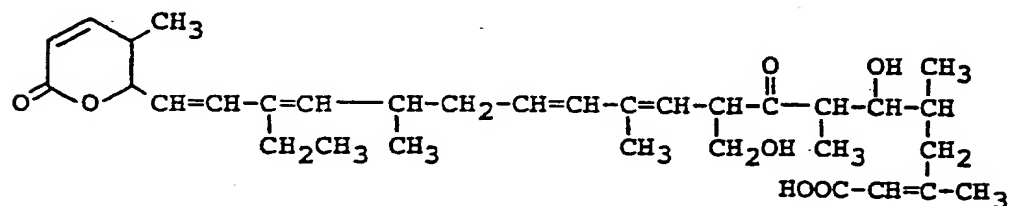
Property	CL-1957b
Molecular weight	556 atomic mass units
Elemental analysis ^a	Calculated for $C_{33}H_{48}O_7 \cdot 0.32 CHCl_3$: 67.28% C, 8.13% H, 5.73% Cl. Found: 66.92% C, 8.21% H, 5.62% Cl.
Melting point	49°-52°C (with prior softening)
Optical rotation	$[\alpha]_D^{23} = -157^\circ$ (0.7% in chloroform)
Ultraviolet absorption spectra	Free acid form (in methanol): Absorption maximum at 289 nm ($\epsilon = 0.33$); and absorption below 260 nm. Carboxylate form (in methanol): Maxima at 240 nm (inflection), 280 nm ($\epsilon = 0.75$), and 385 nm ($\epsilon = 0.24$).
Infrared absorption spectrum (in chloroform)	Principal absorption peaks at 2970, 2940, 1715, 1700 (shoulder), 1640, 1455, 1375, 1250, 1100, and 965 reciprocal centimeters.
360 MHz proton magnetic resonance spectrum (deuteriochloroform solution)	Principal signals at 0.76 (doublet, 3 protons), 0.95 (doublet, 3 protons), 1.03 (triplet, 3 protons), 1.05 (doublet, 3 protons), 1.17 (doublet, 3 protons), 1.74 (multiplet, 1 proton), 1.84 (singlet, 3 protons), 1.91 (doublet of doublets, 1 proton), 2.06 (multiplet, 2 protons), 2.11 (singlet, 3 protons), 2.15 (multiplet, 1 proton), 2.18 (quartet, 2 protons), 2.52 (multiplet, 1 proton), 2.65 (multiplet, 1 proton), 2.78 (multiplet, 1 proton), 3.60 (multiplet, 2 protons), 3.05 (multiplet, 2 protons), 4.96 (doublet of doublets, 1 proton), 5.02 (doublet, 1 proton), 5.20 (doublet, 1 proton), 5.61 (doublet of doublets, 1 proton), 5.66 (singlet, 1 proton), 5.69 (doublet of doublets, 1 proton), 5.98 (doublet, 1 proton), 5.99 (doublet, 1 proton), 6.61 (doublet, 1 proton), and 6.93 (doublet of doublets, 1 proton) parts per million downfield from tetramethylsilane.

TABLE IX, continued.
Chemical and Physical Properties of CL-1957B

Property	CL-1957B
90.5 MHz ^{13}C nuclear magnetic resonance spectrum (deuteriochloroform solution)	Principal signals at 214.97, 170.60, 164.40, 160.95, 151.97, 139.36, 136.80, 135.62, 134.90, 130.26, 128.90, 122.69, 122.08, 120.03, 116.81, 81.55, 73.99, 62.61, 53.84, 47.96, 45.66, 40.82, 33.64, 33.56, 32.22, 26.61, 20.92, 18.67, 13.63, 13.58, 13.33, 12.39, and 12.32 parts per million downfield from tetramethylsilane.
Retention time (high pressure chromatography, μ Bondapak (TM) C18-silica gel column, 3.9 mm I.D. x 30 cm, Waters Associates, Milford, MA, solvent: 45:55 0.05 M ammonium acetate buffer (pH 6.5)-acetonitrile, flow rate 1.5 ml/min)	4.00 minutes
R _f (thin-layer chromatography on silica gel 60 F254, E. Merck, solvent 90:10 chloroform-methanol)	0.33

*Elemental analysis calculated on the basis of included chloroform solvent of crystallization

While not holding to particular structures to the exclusion of others, the chemical structure of CL-1957B is believed to correspond to that indicated by structure I below, which is consistent with the
 5 spectral data presented in Table IX.



I (CL-1957B)

The exact cis-trans configuration of the groups attached to the lactone ring and the exact E-Z configurations about the carbon-carbon double bonds are not known with certainty at the time of filing of
 10 the present application. The present invention is therefore contemplated as encompassing all possible cis-trans and E-Z isomers of structure I given above.
 15 The name of the compound designated above (while not specifying cis-trans or E-Z configuration) is 19-(3,6-dihydro-3-methyl-6-oxo-2H-pyran-2-yl)-

17-ethyl-6-hydroxy-9-(hydroxymethyl)-3,5,7,11,
15-pentamethyl-8-oxo-2,10,12,16,18-nonadecapentaenoic
acid.

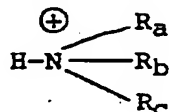
The compound of the present invention forms
5 pharmaceutically acceptable salts with organic and
inorganic bases. Examples of suitable inorganic bases
are ammonium hydroxide, sodium hydroxide, potassium
hydroxide, sodium carbonate, calcium hydroxide, sodium
bicarbonate, and the like. Pharmaceutically
10 acceptable salts are also formed with amine cations
derived from organic nitrogenous bases strong enough
to form cations.

The pharmaceutically acceptable salts of the acid
are prepared by, for example, suspending the acid in
15 water and adjusting the pH with the pharmaceutically
acceptable base, or by reacting the compounds with one
equivalent of the pharmaceutically acceptable base in
a solvent and removing the solvent under reduced
pressure.

20 The term, pharmaceutically acceptable metal
cation contemplates the positively charged ions deriv-
ed from such metals as sodium, potassium, calcium,
magnesium, aluminum, zinc, iron, and the like. The
salts are prepared by contacting the free acid form of
25 the compound with an equivalent amount of the desired
base in the conventional manner. The free acid form
may be regenerated by treating the salt form with an
acid. For example, dilute aqueous acid solutions may
be utilized to regenerate the free acid form from a
30 respective salt. Dilute aqueous hydrochloric acid is
suitable for this purpose. The free forms differ from

their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but the salts are otherwise equivalent to their respective free base forms for purposes of the invention.

The term pharmaceutically acceptable amine cation contemplates the positively charged ammonium ion and analogous ions derived from organic nitrogenous bases strong enough to form such cations. Bases useful for the purpose of forming pharmacologically acceptable nontoxic addition salts of such compounds containing free carboxyl groups form a class whose limits are readily understood by those skilled in the art. Merely for illustration, they can be said to comprise, in cationic form, those of the formula:



wherein R_a , R_b , and R_c , independently, are hydrogen, alkyl of from about one to about six carbon atoms, cycloalkyl of from about three to about six carbon atoms, aryl of about six carbon atoms, aralkyl of from about 7 to about 11 carbon atoms, hydroxyalkyl of from about 2 to about 4 carbon atoms, or monoarylhydroxyalkyl of from about 8 to about 15 carbon atoms, or, when taken together with the nitrogen atom to which they are attached, any two of R_a , R_b , and R_c may form part of a 5- to 6-membered heterocyclic ring containing carbon, hydrogen, oxygen, or nitrogen, said heterocyclic rings and said aryl groups being unsubstituted or mono- or dialkyl substituted said alkyl groups containing from about one to about six carbon atoms. Illustrative therefore

of R_a, R_b, and R_c groups comprising pharmaceutically acceptable cations derived from ammonia or a basic amine are ammonium, mono-, di-, and trimethylammonium, mono, di- and triethylammonium, mono-,
5 di-, and tripropylammonium (iso and normal), ethyldimethylammonium, benzyltrimethylammonium, cyclohexylammonium, benzylammonium, dibenzylammonium, piperidinium, morpholinium, pyrrolidinium, piperazinium, pyridinium, 1-methylpiperidinium, 4-ethylmorpholinium,
10 1-isopropylpyrrolidinium, 1,4-dimethylpiperazinium, 1-n-butylpiperidinium, 2-methylpiperidinium, 1-ethyl-2-methylpiperidinium, mono-, di-, and triethanolammonium, ethyldiethanolammonium, n-butylmonoethanolammonium, tris(hydroxymethyl)-
15 methylammonium, phenylmonoethanolammonium, and the like.

Biological Activity of CL-1957B

EXAMPLE 10

The antimicrobial activity of CL-1957B was
20 evaluated by saturating 12.7 mm paper discs with solutions of CL-1957B prepared at concentrations of 10, 100, and 500 µg/ml and placing each saturated paper disc on a bioassay tray containing an agar medium seeded with a particular organism. The disc
25 and inoculated medium were incubated for 16 hours at 37°C and the diameter of the resulting growth inhibition zone, if any, was measured. The data from these tests appear in Table X.

TABLE X

Microorganism	Culture Number*	Medium	Diameter of Inhibition Zone (mm)			
			CL-1957b	500 µg/ml	100 µg/ml	10 µg/ml
<u>Alcaligenes viscolactis</u>	ATCC 21698	Mycin	0	0	0	0
<u>Bacillus subtilis</u>	ATCC 6633	#169	21	0	0	0
<u>Bacillus subtilis</u>	PD 04969	#169	15	0	0	0
<u>Bacillus subtilis</u>	ATCC 6633	Mycin	0	0	0	0
<u>Escherichia coli</u>	ATCC 10536	GAA	0	0	0	0
<u>Klosteria brevis</u>	PD M1378	#69	0	0	0	0
<u>Branhamella catenulalis</u>	PD 03596	CAP	27	14	0	0
<u>Penicillium avellaneum</u>	PD M2988	H & B	0	0	0	0
<u>Protens vulgaris</u>	PD 05062	PAS	0	0	0	0
<u>Micrococcus luteus</u>	PD 05064	PAS	14	0	0	0
<u>Staphylococcus aureus</u>	PD 02482	PAS	20	0	0	0
<u>Staphylococcus aureus</u>	PD 5045	AM-10	28	18	0	0
<u>Staphylococcus aureus</u>	PD 5045	AM-9	26	26	0	0
<u>Xanthomonas phaseoli</u>	PD 06002	CMA	0	0	0	0

*ATCC = American Type Culture Collection, Rockville, Maryland 20852

PD = Warner-Lambert/Packe-Davis Culture Collection, 2800 Plymouth Road, Ann Arbor, Michigan 48105

EXAMPLE 11

The in vivo activity of CL-1957B against P388 against P388 leukemia in mice, was assayed using the protocol established in Cancer Chemotherapy Reports, Vol. 3, Part 3, 1-87 (1972). The mice were infected intraperitoneally on Day 0 and then given the dose of CL-1957B indicated in Table XI on Days 1-5. The results of these tests are presented in Table XI in terms of % T/C values as defined above.

TABLE XI

In Vivo Activity of CL-1957B
Against P388 Leukemia in Mice

CL-1957B Dose (ug/kg/ injection)	% T/C	
	Test-1	Test-2
200	Toxic	Toxic
100	154	Toxic
50	143	161
25	139	148
12.5	111	136

EXAMPLE 12

The cytotoxicity of CL-1957B against L1210 mouse leukemia cells and against human colon adenocarcinoma cells were measured in vitro. The ID₅₀ values appear in Table XII.

TABLE XII

Compound	ID ₅₀	
	L1210 Mouse Leukemia Cells	Human Colon Adenocarcinoma Cells
5 CL-1957B	0.185 ng/ml	0.13 ng/ml

EXAMPLE 13

In this Example, the in vivo activity of CL-1957B was tested against Ridgway Osteogenic Sarcoma in mice as follows. Male AKR mice were pooled, inoculated
 10 subcutaneously by trocar with 30 to 60 mg fragments of Ridgway Osteogenic Sarcoma, repooled, and randomly distributed to treat groups on Day 0.

Appropriate mice received intraperitoneal injections of the test compound dissolved in 0.9% sodium
 15 chloride solution on Days 2, 6, and 10, and then weekly thereafter. Tumors were measured on Days 24 and 35. The results are presented in Table XIII as % T/C (as defined below.) % T/C values of less than 40 on Day 35 are considered active.

$$20 \quad \% \text{ T/C} = \frac{\text{Tumor size of test animals}}{\text{Tumor size of control animals}} \times 100$$

TABLE XIII

Activity of CL-1957B Against Ridgway Osteogenic
Sarcoma in Mice

5	Dose (mg/kg of body weight/injection)	% T/C	
		Day 24	Day 35
	0.375	11	17
	0.188	30	54

EXAMPLE 14

- The in vivo activity of CL-1957B against B16 melanoma in mice was tested using the protocol established in Cancer Chemotherapy Reports, Vol. 3, Part 3, 1-87 (1972). Mice were inoculated by trochar with B16 melanoma on Day 0 and then given intraperitoneal injections of CL-1957B on Days 1, 5, and 9.
- 15 The activity of the compound against B16 melanoma is present in Table XIV in terms of % T/C values which represents the ratio of median life span in days of treated mice to untreated mice, expressed as a percentage.

TABLE XIV

Activity of CL-1957B Against B16 Melanoma
in Mice

5	Dose	% T/C
	(mg/kg of body weight/injection)	
	0.75	18
	0.375	141
	0.188	141
	0.094	151

- 10 The antimicrobial compound CL-1957B, either in
its free acid form, or in the form of one or more of
its pharmaceutically acceptable salts, is useful for
its antimicrobial and antitumor activity as pharmaceu-
15 tical compositions in combination with a compatible
pharmaceutically acceptable carrier. These
compositions may also contain other antimicrobial
and/or antitumor agents. The compositions may be made
up in any pharmaceutically appropriate form for the
desired route of administration. Examples of such
20 forms include solid forms for oral administration as
tablets, capsules, pills, powders and granules, liquid
forms for topical or oral administration as solutions,
suspensions, syrups, and elixirs, and forms suitable
for parenteral administration such as sterile
25 solutions, suspensions, or emulsions.

For preparing pharmaceutical compositions from
the compounds described by this invention, inert,
pharmaceutically acceptable carriers can be either
solid or liquid. Solid form preparations include
30 powders, tablets, dispersible granules, capsules,
cachets, and suppositories. A solid carrier can be

one or more substances which may also act as diluents, flavoring agents, solubilizers, lubricants, suspending agents, binders, or tablet disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active compound. In the tablet the active compound is mixed with carrier having the necessary binding properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain from 5 or 10 to about 70 percent of the active ingredient. Suitable solid carriers are magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter, and the like. The term "preparation" is intended to include the formulation of the active compound with encapsulating material as carrier providing a capsule in which the active component with encapsulating material as carrier providing a capsule in which the active component (with or without other carriers) is surrounded by carrier, which is thus in association with it. Similarly, cachets are included. Tablets, powders, cachets, and capsules can be used as solid dosage forms suitable for oral administration.

For preparing suppositories, a low melting wax such as a mixture of fatty acid glycerides or cocoa butter is first melted, and the active ingredient is dispersed homogeneously therein as by stirring. The molten homogeneous mixture is then poured into convenient sized molds, allowed to cool and thereby to solidify.

Liquid form preparations include solutions, suspensions, and emulsions. As an example may be mentioned water or water-propylene glycol solutions for parenteral injection. Liquid preparations can
5 also be formulated in solution in aqueous polyethylene glycol solution. Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding suitable colorants, flavors, stabilizing, and thickening agents as desired.
10 Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, i.e., natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known
15 suspending agents.

Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations for either oral or parenteral administration. Such liquid forms include solutions,
20 suspensions, and emulsions. These particular solid form preparations are most conveniently provided in unit dose form and as such are used to provide a single liquid dosage unit. Alternately, sufficient solid may be provided so that after conversion to
25 liquid form, multiple individual liquid doses may be obtained by measuring predetermined volumes of the liquid form preparation as with a syringe, teaspoon, or other volumetric container. When multiple liquid doses are so prepared, it is preferred to maintain
30 the unused portion of said liquid doses at low temperature (i.e., under refrigeration) in order to retard possible decomposition. The solid form preparations intended to be converted to liquid form may

contain, in addition to the active material, flavor-
ants, colorants, stabilizers, buffers, artificial
and natural sweeteners, dispersants, thickeners,
solubilizing agents, and the like. The liquid
5 utilized for preparing the liquid form preparation
may be water, isotonic water, ethanol, glycerine,
propylene glycol, and the like as well as mixtures
thereof. Naturally, the liquid utilized will be
chosen with regard to the route of administration,
10 for example, liquid preparations containing large
amounts of ethanol are not suitable for parenteral
use.

Preferably, the pharmaceutical preparation is in
unit dosage form. In such form, the preparation is
15 subdivided into unit doses containing appropriate
quantities of the active component. The unit dosage
form can be a packaged preparation, the package con-
taining discrete quantities of preparation, for
example, packeted tablets, capsules, and powders in
20 vials or ampoules. The unit dosage form can also
be a capsule, cachet, or tablet itself or it can be
the appropriate number of any of these in packaged
form.

The quantity of active compound in a unit dose
25 of preparation may be varied or adjusted from 0.1 mg
to 500 mg preferably to 5 to 100 mg according to the
particular application and the potency of the active
ingredient. The compositions can, if desired, also
contain other compatible therapeutic agents.

30 In therapeutic use, the mammalian dosage range
for a 70 kg subject is from 1 to 1500 mg/kg of body
weight per day or preferably 2 to 750 mg/kg of body
weight per day. The dosages, however, may be varied
depending upon the requirements of the patient, the

severity of the condition being treated, and the compound being employed. Determination of the proper dosage for a particular situation is within the skill of the art. Generally, treatment is initiated with
5 smaller dosages which are less than the optimum dose of the compound. Thereafter the dosage is increased by small increments until the optimum effect under the circumstances is reached. For convenience, the total daily dosage may be divided and administered in
10 portions during the day if desired.

CLAIMS (for the Contracting States: BE, CH, DE, FR, GB, IT, LI, LU, NL, SE)

1. A compound designated CL-1957B, and the pharmaceutically acceptable salts thereof, wherein compound CL-1957B is characterised by:
- 5 a) a molecular weight of 556 atomic mass units;
- b) a melting point of 49-52°C (with prior softening);
- 10 c) an optical rotation, $[\alpha]_D^{23}$, of -156° (0.82% in chloroform);
- d) an ultraviolet absorption spectrum in methanol (free acid form) showing an absorption maximum at 289 nm ($a = 0.33$) and end absorption below 260 nm;
- 15 e) an ultraviolet absorption spectrum in methanol (carboxylate anion form) showing maxima at 240 nm ($a = 0.75$), and 385 nm ($a = 0.24$);
- 20 f) an infrared spectrum in chloroform solution showing principal absorption peaks at 2970, 2940, 1715, 1700 (shoulder), 1640, 1455, 1375, 1250, 1100 and 965 reciprocal centimetres;
- 25 g) a 360 MHz proton magnetic resonance spectrum in deuterochloroform solution showing signals at 0.76 (doublet, 3 protons), 0.95 (doublet, 3 protons), 1.03 (triplet, 3 protons), 1.05 (doublet, 3 protons), 1.17 (doublet, 3 protons), 1.74 (multiplet, 1 proton), 1.84 (singlet, 3 protons), 1.91 (doublet of doublets, 1 proton), 2.06 (multiplet, 2 protons), 2.11 (singlet, 3 protons), 2.15 (multiplet, 1 proton), 2.18 (quartet, 2 protons), 2.52 (multiplet, 1 proton), 2.65 (multiplet, 1 proton), 2.78 (multiplet, 1 proton),
- 30
- 35

3.60 (multiplet, 2 protons), 3.85
(multiplet, 2 protons), 4.96 (doublet
of doublets, 1 proton), 5.02 (doublet,
1 proton), 5.20 (doublet, 1 proton),
5.61 (doublet of doublets, 1 proton),
5.66 (singlet, 1 proton), 5.69 (doublet
of doublets, 1 proton), 5.98 (doublet,
1 proton), 5.99 (doublet, 1 proton),
and 6.93 (doublet of doublets, 1 proton)
parts per million downfield from
tetramethylsilane; and
h) a 90.5 MHz ¹³C nuclear magnetic resonance
spectrum in deuteriochloroform solution
principal signals at 214.97, 170.60,
164.40, 160.95, 151.97, 139.36, 136.80,
135.62, 134.90, 130.26, 138.90, 122.69,
122.08, 120.03, 116.81, 81.55, 73.99,
62.61, 53.84, 47.96, 45.66, 40.82, 33.64,
33.56, 32.22, 26.61, 20.92, 18.67, 13.63,
13.58, 13.33, 12.39, 12.32 parts per
million downfield from tetramethylsilane.

2. The stereochemical isomers of 19-
(3,6-dihydro-3-methyl-6-oxo-2H-pyran-2-yl)-17-
ethyl-6-hydroxy-9-(hydroxymethyl)-3,5,7,11,15-
pentamethyl-8-oxo-2,10,12,16,18-nonadecapentaenoic
acid and the pharmaceutically acceptable salts
thereof.

3. The compound designated CL-1957B as
defined in Claim 1, and the pharmaceutically
acceptable salts thereof.

4. A pharmaceutical composition comprising
at least one compound as claimed in Claim 2,
together with a pharmaceutically acceptable carrier.

5. A pharmaceutical composition comprising
at least one compound designated CL-1957B as
claimed in Claim 1, together with a pharmaceutically
acceptable carrier.

6. A process for the production of CL-1957B, which comprises cultivating a strain of an actinomycete, identified as isolate ATCC 39366, under aerobic conditions in a culture medium containing assimilable sources of carbon and nitrogen until a substantial amount of CL-1957B is produced and subsequently isolating said compound.

7. A process for the production of the compound 19-(3,6-dihydro-3-methyl-6-oxo-2H-pyran-2-yl)-17-ethyl-6-hydroxy-9-(hydroxymethyl)-3,5,7,11,15-pentamethyl-8-oxo-2,10,12,16,18-nonadecapentaenoic acid, which process comprises cultivating a strain of an actinomycete, identified as isolate ATCC 39366, under aerobic conditions in a culture medium containing assimilable sources of carbon and nitrogen until a substantial amount of the compound is produced and subsequently isolating said compound.

8. A purified isolate of an actinomycete having the identifying characteristics of ATCC 39366, which isolate is capable of producing the antibiotic CL-1957B compound under aerobic fermentation in a culture medium containing assimilable sources of carbon and nitrogen.

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CLAIMS (for the Contracting State: AT)

1. A process for producing a compound designated CL-1957B, wherein compound CL-1957B is characterised by:

- 5 a) a molecular weight of 556 atomic mass units;
- b) a melting point of 49-52°C (with prior softening);
- 10 c) an optical rotation, $[\alpha]_D^{23}$, of -156° (0.82% in chloroform);
- d) an ultraviolet absorption spectrum in methanol (free acid form) showing an absorption maximum at 289 nm ($a = 0.33$) and end absorption below 260 nm;
- 15 e) an ultraviolet absorption spectrum in methanol (carboxylate anion form) showing maxima at 240 nm ($a = 0.75$), and 385 nm ($a = 0.24$);
- 20 f) an infrared spectrum in chloroform solution showing principal absorption peaks at 2970, 2940, 1715, 1700 (shoulder), 1640, 1455, 1375, 1250, 1100 and 965 reciprocal centimetres;
- 25 g) a 360 MHz proton magnetic resonance spectrum in deuterochloroform solution showing signals at 0.76 (doublet, 3 protons), 0.95 (doublet, 3 protons), 1.03 (triplet, 3 protons), 1.05 (doublet, 3 protons), 1.17 (doublet, 3 protons), 1.74 (multiplet, 1 proton), 1.84 (singlet, 3 protons), 1.91 (doublet of doublets, 1 proton), 2.06 (multiplet, 2 protons), 2.11 (singlet, 3 protons), 2.15 (multiplet, 1 proton), 2.18 (quartet, 2 protons), 2.52 (multiplet, 1 proton), 2.65 (multiplet, 30 1 proton), 2.78 (multiplet, 1 proton), 35

3.60 (multiplet, 2 protons), 3.85 (multiplet, 2 protons), 4.96 (doublet of doublets, 1 proton), 5.02 (doublet, 1 proton), 5.20 (doublet, 1 proton), 5.61 (doublet of doublets, 1 proton), 5.66 (singlet, 1 proton), 5.69 (doublet of doublets, 1 proton), 5.98 (doublet, 1 proton), 5.99 (doublet, 1 proton), and 6.93 (doublet of doublets, 1 proton) parts per million downfield from tetramethylsilane; and

h) a 90.5 MHz ^{13}C nuclear magnetic resonance spectrum in deuteriochloroform solution principal signals at 214.97, 170.60, 164.40, 160.95, 151.97, 139.36, 136.80, 135.62, 134.90, 130.26, 138.90, 122.69, 122.08, 120.03, 116.81, 81.55, 73.99, 62.61, 53.84, 47.96, 45.66, 40.82, 33.64, 33.56, 32.22, 26.61, 20.92, 18.67, 13.63, 13.58, 13.33, 12.39, 12.32 parts per million downfield from tetramethylsilane;

which process comprises cultivating a strain of an actinomycete, identified as isolate ATCC 39366, under aerobic conditions in a culture medium containing assimilable sources of carbon and nitrogen until a substantial amount of Cl-1957B is produced, subsequently isolating said compound, and optionally forming a pharmaceutically acceptable salt thereof.

2. A process for producing the stereochemical isomers of 19-(3,6-dihydro-3-methyl-6-oxo-2H-pyran-2-yl)-17-ethyl-6-hydroxy-9-(hydroxymethyl)-3,5,7,11,15-pentamethyl-8-oxo-2,10,12,16,18-nonadecapentaenoic acid and the pharmaceutically acceptable salts thereof;

which process comprises cultivating a strain of an actinomycete, identified as isolate ATCC 39366, under aerobic conditions in a culture medium containing assimilable sources of carbon and nitrogen until a substantial amount of the compound is produced, subsequently isolating said compound, and optionally forming a pharmaceutically acceptable salt.

3. A process according to Claim 1 for producing the compound designated CL-1957B as defined in Claim 1.

4. A process for preparing a pharmaceutical composition which process comprises combining the compound designated
5 CL-1957B, produced in accordance with Claim 1, together with a pharmaceutically acceptable carrier.

5. A process for preparing a pharmaceutical composition which process comprises combining the compound 19-(3,6-dihydro-3-methyl-6-oxo-2H-
10 pyran-2-yl)-17-ethyl-6-hydroxy-9-(hydroxymethyl)-3,5,7,11,15-pentamethyl-8-oxo-2,10,12,16,18-nonadecapentaenoic acid, produced in accordance with Claim 2, together with a pharmaceutically acceptable carrier.

15 6. A purified isolate of an actinomycete having the identifying characteristics of ATCC 39366, which isolate is capable of producing the antibiotic CL-1957B compound under aerobic fermentation in a culture medium containing
20 assimilable sources of carbon and nitrogen.

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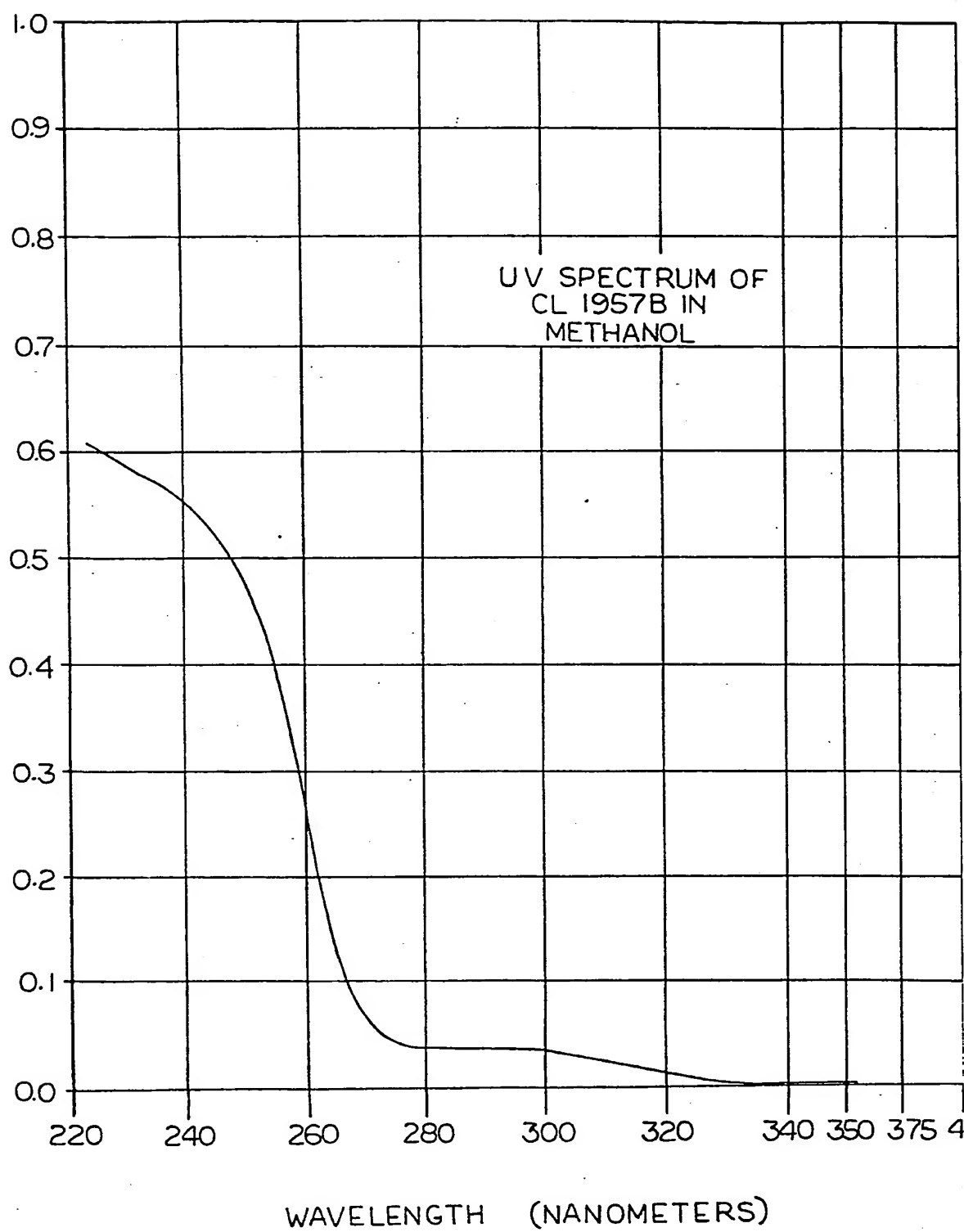


fig 1a

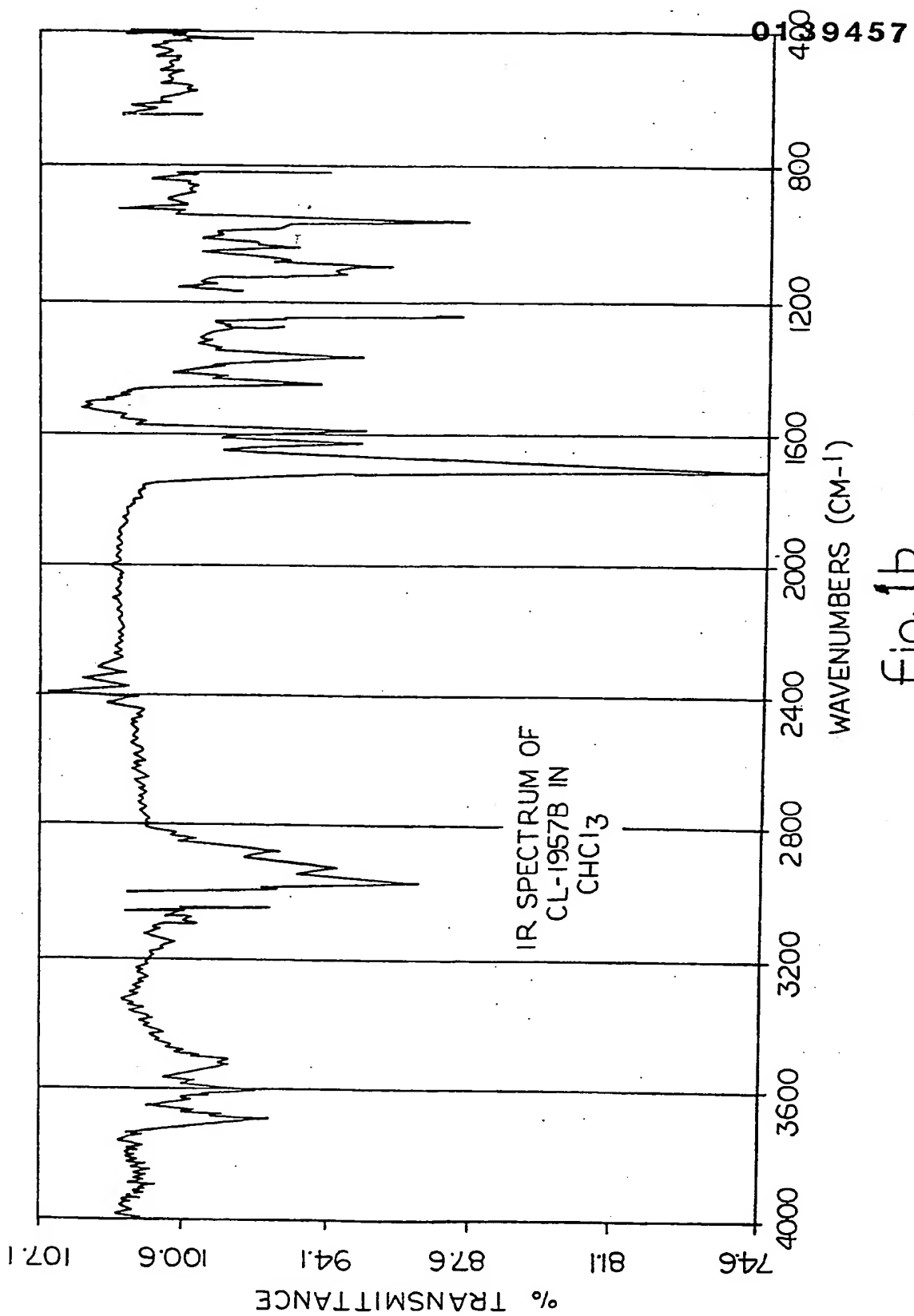
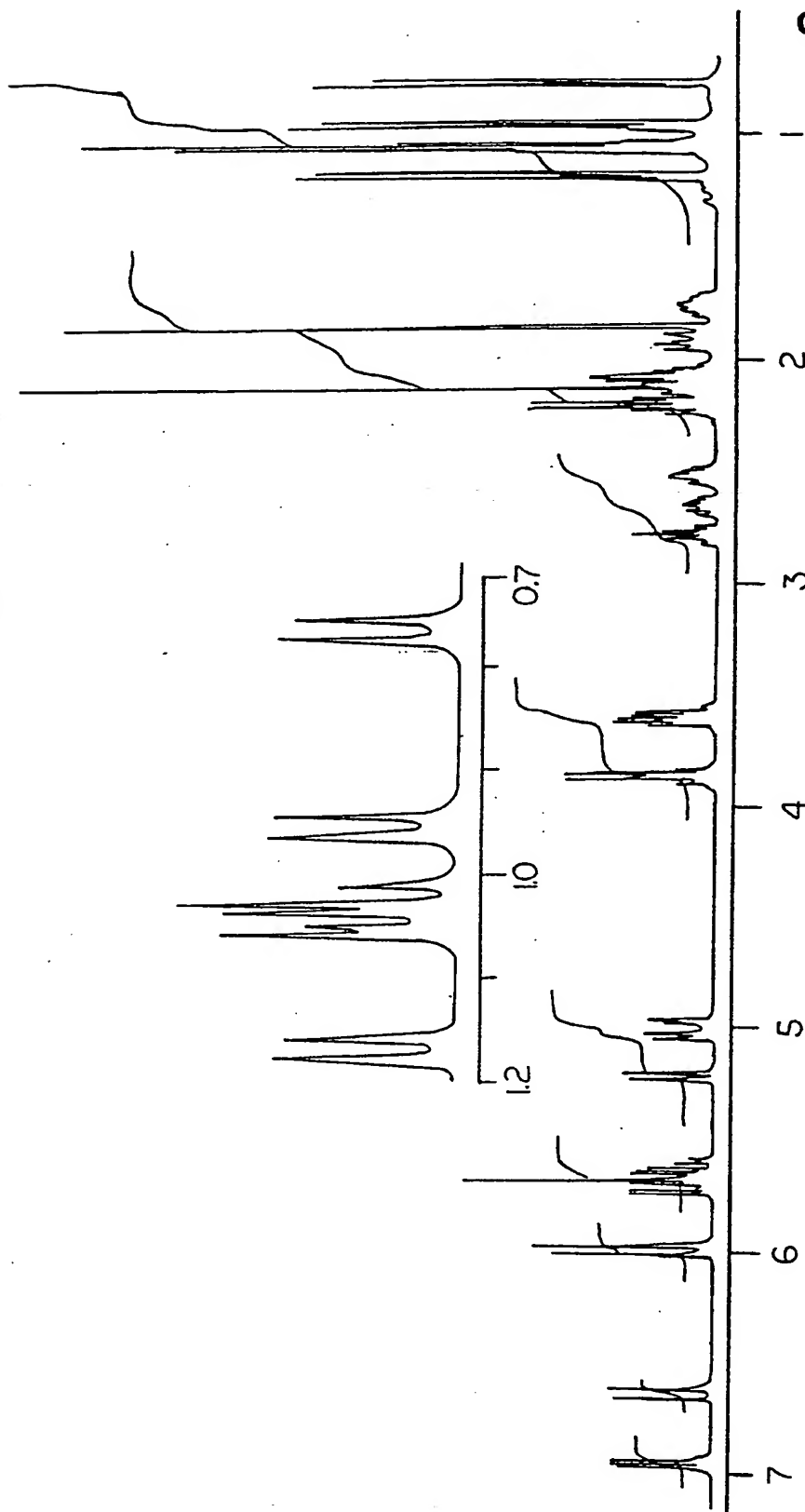


Fig. 1b

360 MHz PMR
SPECTRUM OF CL-1957B
IN D₂O



0139457

Fig. 1c

90.5 MHz ^{13}C -NMR SPECTRUM
OF CL-1957B

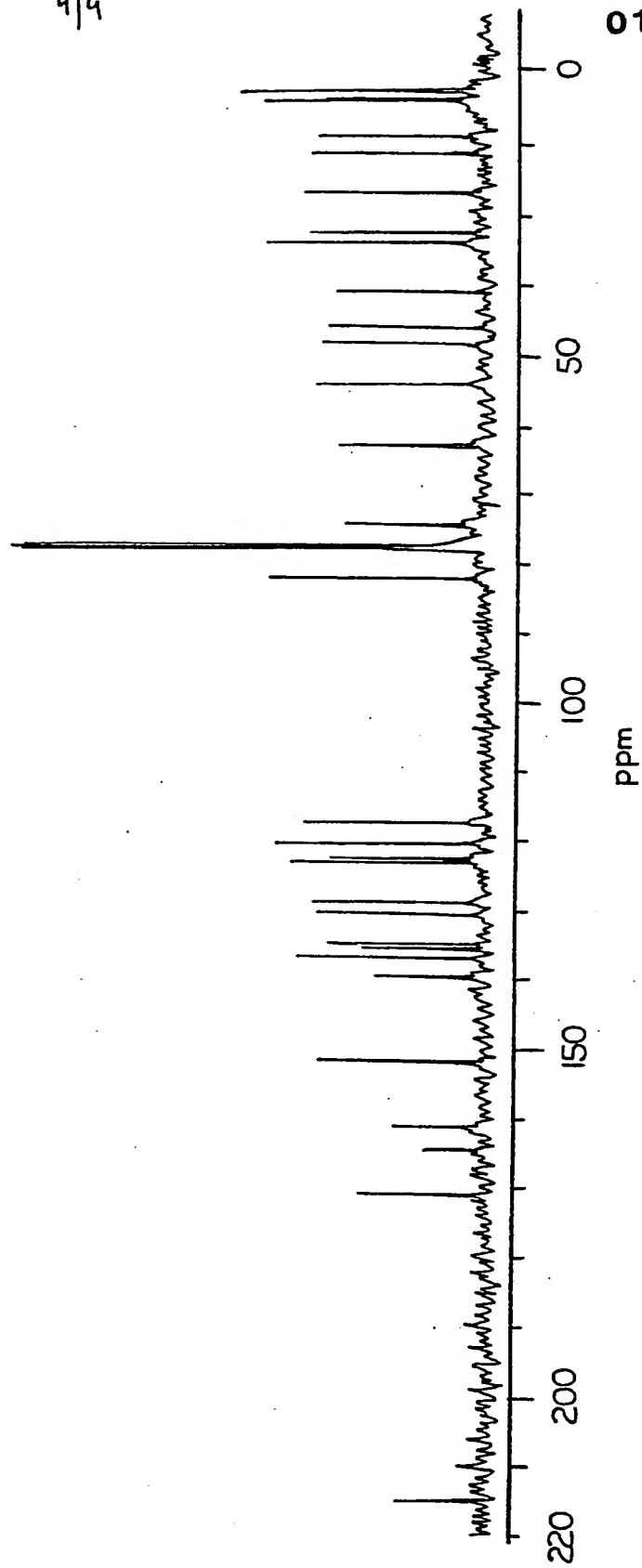


Fig. 1d



European Patent Office

Application number:

01394.

**DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4,
OF THE EUROPEAN PATENT CONVENTION**

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits: **ATCC 39366**

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